The EMBO Journal Vol.17 No.1 pp.244–254, 1998

Binding of the winged-helix transcription factor HNF3 to a linker histone site on the nucleosome

Lisa A.Cirillo, Clifton E.McPherson, Pascale Bossard, Kimberly Stevens, Sindhu Cherian, Eun Yong Shim, Kirk L.Clark[1], Stephen K.Burley[1] and Kenneth S.Zaret[2]

Department of Molecular Biology, Cell Biology and Biochemistry, Brown University, Box G-J363, Providence, RI 02912 and [1]Laboratories of Molecular Biophysics, The Rockefeller University and the Howard Hughes Medical Institute, 1230 York Avenue, New York, NY 10021, USA

[2]Corresponding author
e-mail: zaret@brown.edu

L.A.Cirillo and C.E.McPherson contributed equally to this work

The transcription factor HNF3 and linker histones H1 and H5 possess winged-helix DNA-binding domains, yet HNF3 and other fork head-related proteins activate genes during development whereas linker histones compact DNA in chromatin and repress gene expression. We compared how the two classes of factors interact with chromatin templates and found that HNF3 binds DNA at the side of nucleosome cores, similarly to what has been reported for linker histone. A nucleosome structural binding site for HNF3 is occupied at the albumin transcriptional enhancer in active and potentially active chromatin, but not in inactive chromatin *in vivo*. While wild-type HNF3 protein does not compact DNA extending from the nucleosome, as does linker histone, site-directed mutants of HNF3 can compact nucleosomal DNA if they contain basic amino acids at positions previously shown to be essential for nucleosomal DNA compaction by linker histones. The results illustrate how transcription factors can possess special nucleosome-binding activities that are not predicted from studies of factor interactions with free DNA.

Keywords: chromatin/HNF3/linker histone/nucleosome/transcription factor

Introduction

Understanding how genes are activated in a chromatin context requires investigation of the mechanisms by which transcription factors interact with nucleosomes. Nucleosome cores contain ~146 bp of DNA wrapped nearly twice around an octamer of core histone proteins; this repeating unit of chromatin is bound further by a molecule of linker histone, which compacts DNA on the particle (reviewed by Zlatanova and van Holde, 1996). The ability to reconstitute nucleosomes *in vitro* with defined nucleotide sequences has allowed a detailed analysis of nucleosome interactions with transcription factors (Perlmann and Wrange, 1988; Piña *et al.*, 1990; Workman and Kingston, 1992; Lee *et al.*, 1993; Wechsler *et al.*, 1994; Alevizopoulos *et al.*, 1995; Patterton and Hapgood, 1996). These studies have typically been guided by first knowing where transcription factors bind their target sites on free DNA, and have revealed that all known factors bind with lower affinities to nucleosomes than to free DNA (reviewed by Adams and Workman, 1995). However, it remains possible that investigating factors binding to nucleosome templates *in vitro* may reveal sites that are bound with equal or better affinity than on free DNA, and where nucleosome structure, in addition to DNA sequence, is important. Herein we describe one such class of sites for a transcription factor containing the 'winged-helix' DNA-binding domain.

The winged-helix domain constitutes the DNA-binding segment of diverse proteins that interact with chromatin. The domain was named for the crystal structure of the liver-enriched transcription factor HNF3γ (Clark *et al.*, 1993), which is a variant of a helix–turn–helix protein (reviewed by Patikoglou and Burley, 1997). HNF3γ binds DNA as a monomer by using a recognition helix flanked by two 'wings' of polypeptide chain interacting with one face of the DNA (see Figure 6 below). The HNF3α, -β and -γ proteins contain 93% sequence identity within their respective 110 amino acid winged-helix domains (Lai *et al.*, 1991) and 90% identity with the winged-helix domain of the fork head protein in *Drosophila* (Weigel *et al.*, 1989). The HNF3 and fork head proteins activate genes during gut development in mammals and flies, respectively, and constitute a subgroup of the large family of fork head-like transcription factors that regulate genes in various developmental contexts (Kaufmann and Knöchel, 1996). Interestingly, the central globular domain of linker histone H5, found in chicken erythrocytes, and the ubiquitous linker histone H1 exhibit a winged-helix structure which is remarkably similar to that of HNF3γ except for the lack of a second wing (Ramakrishnan *et al.*, 1993; Cerf *et al.*, 1994). Both linker histones condense chromatin and repress gene activity (van Holde, 1989), and their ability to compact DNA on the nucleosome is dependent upon basic amino acids opposite the primary DNA-binding 'face' of the globular domain surface (Goytisolo *et al.*, 1996).

Previous studies of linker histones showed that the winged-helix domain was sufficient for nucleosome core binding (Allan *et al.*, 1980). DNase I footprinting studies of linker histones binding to mixed sequence dinucleosomes showed that linker histones protect a region near the dyad axis of the core particle (Staynov and Crane-Robinson, 1988). However, cross-linking studies of bulk chromatin (Bavykin *et al.*, 1990) and

of nucleosomes composed of a unique 5S rRNA gene sequence showed that histones H1 and H5 can bind asymmetrically at one side, or edge, of the nucleosome core (Hayes and Wolffe, 1993; Hayes *et al.*, 1994; Hayes, 1996), with their winged-helix domains probably interacting with both DNA and core histones (Pruss *et al.*, 1995, 1996). We therefore considered the possibility that a winged-helix transcription factor, such as HNF3, might have nucleosome-binding properties like linker histone.

Studies of the mouse serum albumin transcriptional enhancer suggest that HNF3 modulates chromatin structure (Zaret, 1995). The albumin enhancer is liver-specific (Pinkert *et al.*, 1987) and binds HNF3 isoforms, which are liver-enriched, at the eG and eH footprint sites; HNF3 binding to both of these sites is necessary for transcriptional enhancement (Liu *et al.*, 1991; Jackson *et al.*, 1993; Hu and Isom, 1994). *In vivo* footprinting has shown that, in development, the eG HNF3 site is occupied in gut endoderm (Gualdi *et al.*, 1996); endoderm is a precursor to the liver which expresses HNF3 (Ang *et al.*, 1993; Monaghan *et al.*, 1993; Sasaki and Hogan, 1994). Upon hepatic specification, five factors bind the albumin enhancer at sites adjacent to eG, including HNF3 at eH, and the albumin gene is activated (Cascio and Zaret, 1991; Gualdi *et al.*, 1996). Thus, HNF3 binding marks the initial opening of enhancer chromatin in precursor cells and is essential for enhancer activity during differentiation.

Chromatin structure analysis of adult liver nuclei showed that the albumin enhancer exists in an array of three nucleosome-like particles (McPherson *et al.*, 1993). HNF3 and other transcription factors occupy their DNA-binding sites in the context of one of these particles, designated N1, which spans 180 bp of DNA. While it is presently unknown whether the N1 particle contains core histones or is an aggregate of transcription factors bound to DNA, positioning of a nucleosome over the N1 segment occurs *in vitro* when plasmids bearing the albumin enhancer are assembled into chromatin with a *Drosophila* embryo extract (McPherson *et al.*, 1993). Mutation of the eG HNF3 site blocks binding of fork head-like proteins in the fly extract and inhibits nucleosome positioning over eG, implicating HNF3/fork head proteins in chromatin organization (McPherson *et al.*, 1993). These findings from studies of complex chromatin have led us to investigate the nucleosome-binding activity of HNF3 using a completely defined biochemical system.

In the experiments described herein, we compare the binding of HNF3 and linker histones to mononucleosome core particles containing the albumin enhancer N1 sequence. We reveal a binding site for HNF3 on the side of *in vitro* assembled N1 mononucleosome cores, as well as on the albumin N1 particle in intact liver cells and their developmental precursors. We further investigate the ability of wild-type and variant HNF3 isoforms to compact DNA on the nucleosome core, in comparison with linker histone. These studies define a novel chromatin-binding activity of transcription factors, they emphasize nucleosome structure in addition to DNA sequence as an essential binding determinant, and suggest that site-specific DNA-binding factors may decompact DNA from the nucleosome.

Results

A nucleosome-binding site (NS-A1) for HNF3 on the serum albumin enhancer

We assembled core particles from purified core histone proteins and 180 bp, end-labeled DNA fragments containing the sequence of the enhancer N1 particle seen in liver nuclei. The eG and eH high-affinity HNF3-binding sites lie near the middle of the DNA, and thus occur at or near the pseudo-dyad axis of the resulting nucleosome cores (Figure 1G). The N1 sequence cores were purified (McPherson *et al.*, 1996) and subjected to DNase I footprinting with full-length HNF3α protein. We previously showed that the albumin enhancer cores *in vitro* assume three rotational positions of DNA, giving rise to a complex pattern of DNase I cleavage which differs from that of free DNA (McPherson *et al.*, 1996). HNF3α bound to the enhancer eH site on the nucleosome cores and gave rise to a hypersensitive DNase I cleavage in the middle of the footprint which is characteristic of HNF3 binding; footprinting of the eG site was weaker and lacked hypersensitivity (Figure 1A, lanes 4 and 5). HNF3α also bound the nucleosomes in an electromobility shift assay, where HNF3–nucleosome core complexes migrated more slowly than HNF3-free DNA complexes, as expected (Figure 2B, compare lanes 1–4 with 5–9). DNase I footprinting of the lowest mobility HNF3–nucleosome core complexes isolated from the electromobility shift assay gave a footprint pattern like that in the direct footprinting experiment in Figure 1A (data not shown). About 10-fold more HNF3α was required to bind the same amount of nucleosome cores as free enhancer DNA.

Interestingly, an additional HNF3 footprint containing an internal hypersensitive site was observed reproducibly 30–40 bp upstream of the eG site on the nucleosome core particles (Figure 1A, lane 5). We designate the footprint 'nucleosome site-albumin 1' (NS-A1). The NS-A1 footprint occurred at the same HNF3 concentrations which gave specific binding to the eH site on the N1 sequence cores. Occupancy of the NS-A1 site on nucleosome cores and free DNA required the same concentration of HNF3, but the concentration was far greater than that required for eG and eH site binding on free DNA, and resulted in non-specific binding on the free DNA template (Figure 1B, lane 6; dashed lines). Thus, the NS-A1 site was occupied on free DNA only when the DNA was saturated with non-specifically bound protein. To address whether occupancy of HNF3 at eG or eH on the nucleosome cores caused protection at NS-A1, we tested HNF3 binding to nucleosome core templates containing clustered point mutations of the NS-A1 site. The lack of a footprint at NS-A1 on the mutant template (Figure 1C, lanes 2–4) showed that HNF3 binds directly to the NS-A1 site on wild-type templates, and with some sequence specificity. The weaker binding to the eG site on the NS-A1 mutant templates suggests cooperativity between HNF3 at the different sites (see below).

Several lines of evidence indicate that the NS-A1 footprint is due to HNF3α occupying a specific site on a side of the particle, and not linker DNA which could

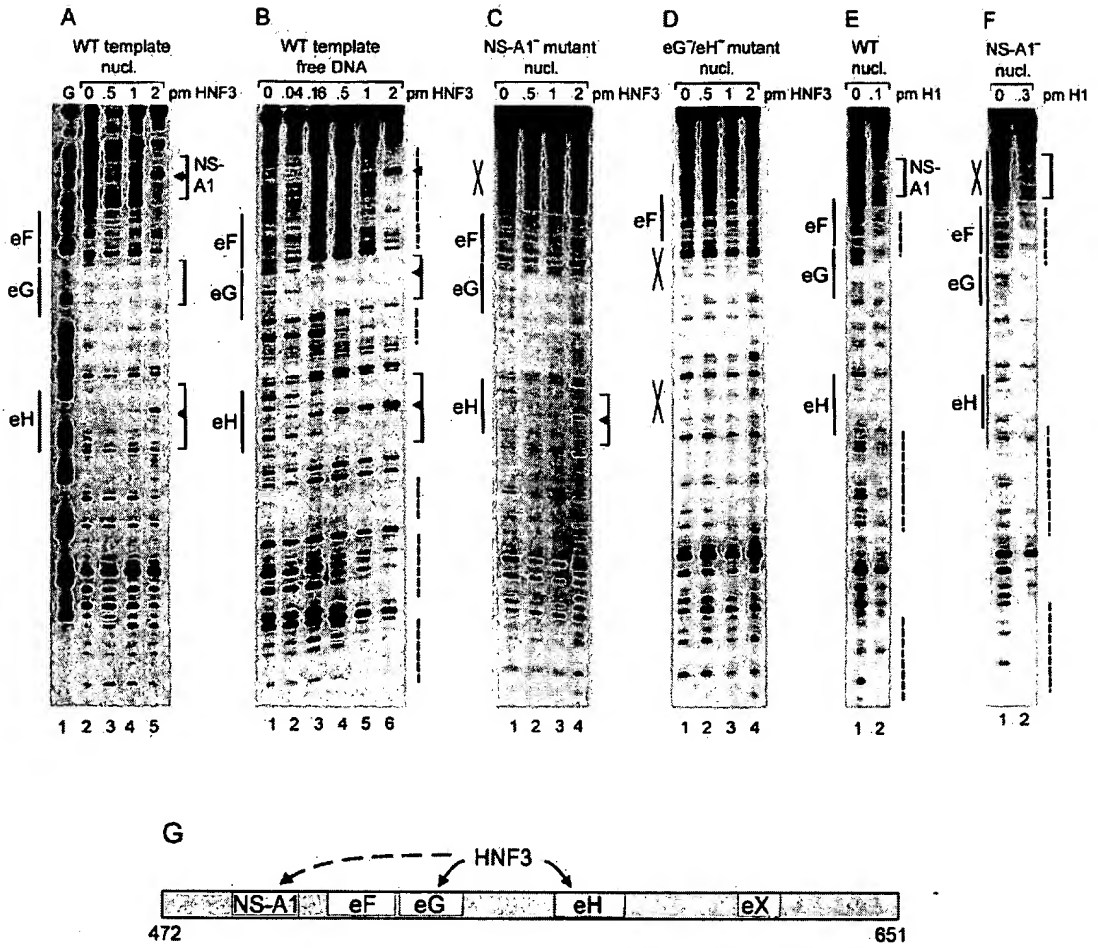

Fig. 1. Nucleosome binding site for HNF3. (A–F) DNase I footprinting analysis of the designated picomole (pm) amounts of HNF3α (**A–D**) or liver histone H1 (**E** and **F**) proteins bound to the designated albumin enhancer templates 5′ end-labeled on the bottom strand. The 20 μl reactions contained 0.42 pmol of free DNA or nucleosomal substrate including a 10-fold ratio of carrier to specific template. The different picomole (pm) amounts of HNF3α or histone H1 (H1) used are shown. The positions of transcription factor-binding sites are indicated at the left of each panel; brackets on the right of each panel indicate protected regions; and dashed lines indicate non-specific protections. Arrowheads within brackets indicate positions of DNase cleavages induced by HNF3 binding. The G lane is a G cleavage ladder size marker; some non-specific cleavages are evident. (G) Summary of HNF3 binding to the 472–651 albumin enhancer fragment (horizontal bar). Labeled boxes indicate transcription factor-binding sites (Liu *et al.*, 1991; McPherson *et al.*, 1993). The region of the nucleosome core dyad axis in (A, C–G) corresponds to that between the eG and eH sites.

extend from the histone core. First, footprinting of additional mutant cores, which contained clustered base changes of the eG and eH sites, showed very little NS-A1 site occupancy (Figure 1D, lanes 2–4). Thus, binding at NS-A1 is cooperative with occupancy at eG and eH. In work to be presented elsewhere, we found that HNF3 binding to eG and eH sites near the nucleosome dyad stabilizes a new position of DNA on the particle (Shim *et al.*, 1998). HNF3 binding near the dyad may result in better positioning of the NS-A1 site and therefore enhance its availability to HNF3. Alternatively, HNF3 binding to the NS-A1 site may be stabilized by another molecule of HNF3 bound to the eH site; the latter is nearly 80 bp downstream and would be one superhelical turn around the histone core from NS-A1. With either explanation, the effects are dependent upon the NS-A1 sequence being bound to the histone core. Second, DNA at the ends of the N1 sequence cores is not protected by HNF3; thus, HNF3 is not invading the edge of the nucleosome via the fragment ends (Figure 1A for the upstream end and data not shown for the downstream end). Third, in an exonuclease III (exoIII) footprinting assay to be described below (Figure 3), binding to the NS-A1 site on free DNA was undetectable at HNF3α concentrations which gave new exoIII boundaries near the NS-A1 site on nucleosome cores. We conclude that HNF3α binds to a new site on the nucleosome core with an affinity comparable with high affinity HNF3-binding sites elsewhere on the particles.

The asymmetric binding of HNF3 to the core particles seemed analogous to what has been proposed for linker histones (see Introduction). We therefore investigated whether mammalian histone H1 recognizes the albumin NS-A1 site on nucleosome cores. Previous studies found that H1 elicits non-specific DNase I protection on mononucleosome cores (Ura *et al.*, 1996), so we carefully titrated H1 concentrations to determine whether preferential protection could be observed. Indeed, we found that 0.1–0.3 pmol of pig liver H1 could elicit relatively strong

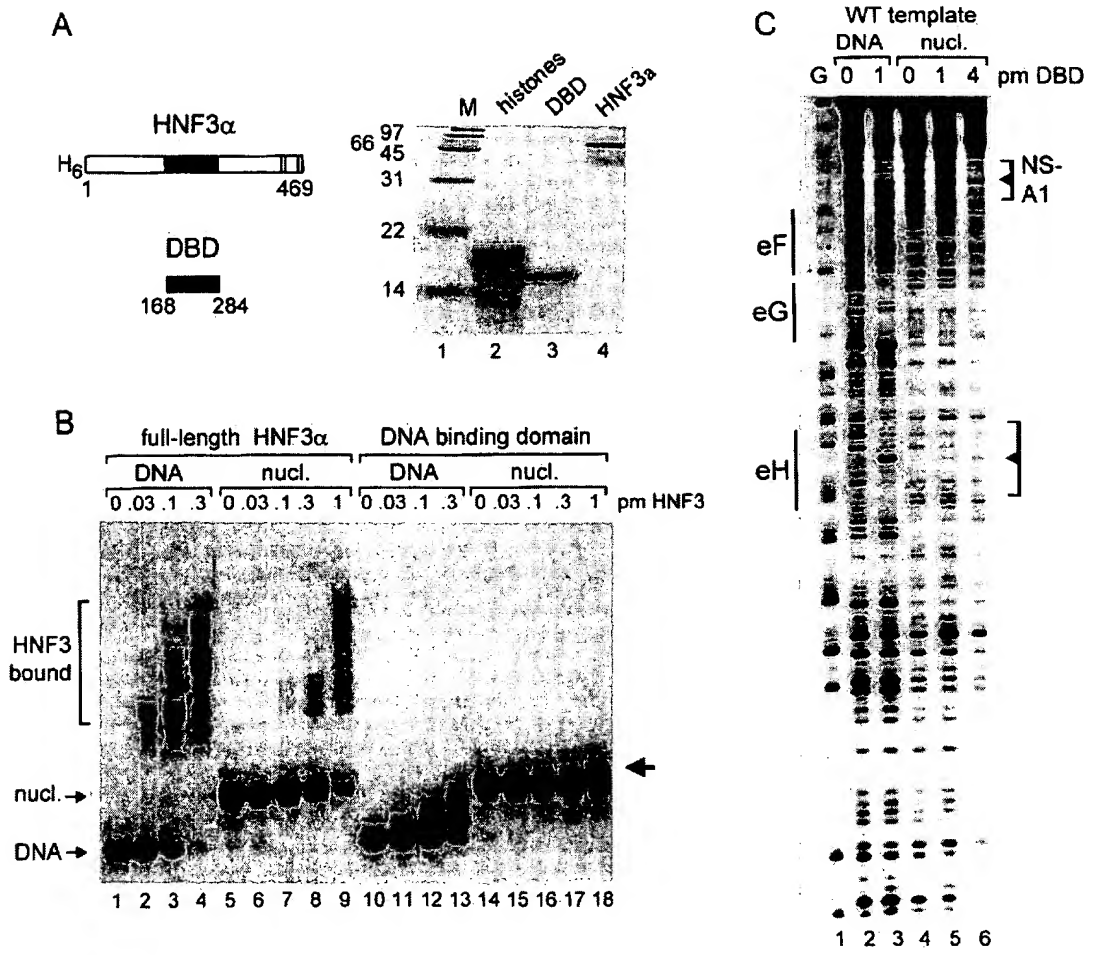

Fig. 2. The winged-helix domain of HNF3α binding to nucleosome cores. (**A**) SDS gel analysis of proteins. Left: schematic view of full-length HNF3α containing a 6× histidine tag and the winged-helix DNA-binding domain (DBD) fragment. Extents of amino acid positions based on the sequences of Lai *et al.* (1991) and Kaestner *et al.* (1994) are shown. Right: 18% polyacrylamide gel analysis of the core histone proteins (20 μg), DBD (5 μg) and HNF3α (4 μg) alongside protein size markers (lane 1; sizes in kilodaltons shown at the side). With this gel system, the middle core histone band contains H2A and H2B superimposed. (**B**) Electromobility shift assay with an end-labeled 180 bp albumin enhancer N1 sequence probe. The positions of free DNA (DNA), nucleosome cores (nucl.) and complexes (HNF3 bound) of different picomole (pm) amounts of HNF3α with free and nucleosomal DNA are indicated. The faint bands migrating faster than the main HNF3-bound complexes in lanes 2–4 and 7–9 are due to binding of a partial HNF3α degradation product (see A, lane 4). The arrow at the right side indicates a DBD–nucleosome complex, which migrates only slightly more slowly than the nucleosome cores. (**C**) DNase I footprinting assay with the designated picomole (pm) amounts of the DBD fragment.

protections over some regions of the particles but not others (Figure 1E, lane 2, dashed lines). The NS-A1 site was among the regions protected (Figure 1E, bracketed region) and the area around the dyad axis, at or between the eG and eH sites, was among the regions exhibiting little or no protection. We also performed the footprinting analysis with albumin enhancer nucleosome cores containing the mutation of the NS-A1 site. Histone H1 still exhibited relatively strong protection in the NS-A1 region and relatively weak protection between the eG and eH sites (Figure 1F). In conclusion, both H1 and HNF3 bind the NS-A1 site as well as other sites that are not the same on the N1 sequence cores. However, H1 recognizes primarily nucleosome core structure at the NS-A1 site whereas HNF3 recognizes both DNA sequence and overall structure.

Other HNF3 protein segments enhance nucleosome binding by the winged-helix domain

To determine if the winged-helix portion of HNF3α is sufficient for nucleosome core binding, we tested the central DNA-binding domain (DBD) of the protein (Figure 2A, lane 3). In electromobility shift assays, the DBD fragment bound free enhancer DNA with an affinity ~70% of that of the full-length HNF3α protein (Figure 2B, compare lanes 1–4 with 10–13). However, the DBD fragment bound nucleosome cores with an affinity ~30% of full-length HNF3α (Figure 2B, compare lanes 5–9 with 14–18). At high protein concentrations, the DBD fragment was capable of eliciting DNase I footprints, albeit weak ones, at the eH and NS-A1 sites (Figure 2C, lanes 5 and 6), along with non-specific DNase protection like that exhibited by linker histone and unlike that of full-length

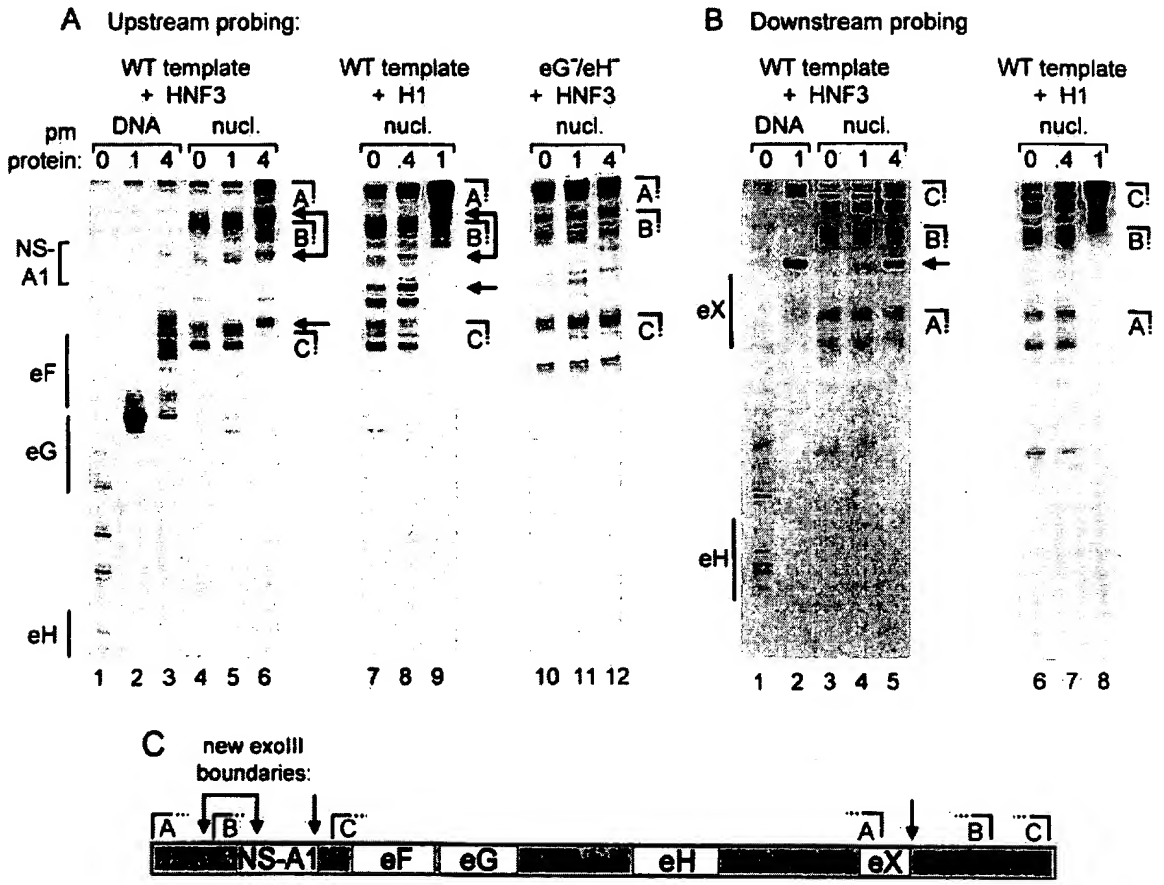

Fig. 3. Changes in exonuclease protection upon HNF3 and histone H1 binding to nucleosome cores. (**A**) Mapping upstream nucleosome boundaries of wild-type (WT) and eG⁻/eH⁻ nucleosome cores (nucl.) and WT free DNA (DNA), 5′ end-labeled on the bottom strand. (**B**) Mapping downstream boundaries with DNA 5′ end-labeled on the top strand. (A and B) Designated amounts of HNF3α or liver histone H1 were incubated with free DNA or nucleosome cores for 30 min and then treated with exoIII. Nucleotide positions were determined by adjacent G cleavage ladders (not shown). Previously identified translational positions (clusters of exoIII cleavage, McPherson *et al.*, 1996) are indicated by brackets labeled A, B and C at the right of each panel. The 'C' boundary presently maps to position 515, which was a minor band in McPherson *et al.* (1996). Arrows indicate new exoIII stops generated by binding of HNF3 or H1. (**C**) Summary of exoIII protection. The brackets labeled A, B and C indicate the previously identified nucleosome core translational positions, and exoIII boundaries induced by HNF3 binding are indicated by arrows above the enhancer.

HNF3α. We conclude that while the DBD can bind to nucleosome cores, amino- and/or carboxy-terminal domains of HNF3α increase its specificity for target sites on nucleosomal but not free DNA.

Asymmetric nucleosome core perturbation by binding of HNF3 or histone H1

Previously we found that when the 180 bp albumin enhancer N1 sequence was assembled into nucleosome cores *in vitro*, the particles exhibited three predominant rotational and translational positions of DNA, designated A, B and C (McPherson *et al.*, 1996; Figure 3C). By contrast, the well-studied 5S rRNA genes and mouse mammary tumor virus (MMTV) promoter each primarily assume a single rotational position on cores *in vitro* (Simpson and Stafford, 1983; Rhodes, 1985; Perlmann and Wrange, 1988; Piña *et al.*, 1990). To determine if HNF3α binding alters nucleosome boundaries, we incubated the protein with N1 sequence cores and treated the products with exoIII. ExoIII digests each strand of DNA in the 3′ to 5′ direction and is impeded by histones or bound transcription factors. Importantly, HNF3α binding did not cause exoIII penetration of the core particles, indicating nucleosome stability during the reactions (Figure 3A and B). Increasing amounts of HNF3α led to reproducible shifts in exoIII protection of the upstream N1 particle boundary B, in the vicinity of the NS-A1 site (Figure 3A, lanes 4–6, arrows). No exoIII stops in this region were induced by the same amounts of HNF3α on free enhancer DNA (Figure 3A, lanes 1–3), providing further evidence that the NS-A1 site is located in nucleosomal and not free DNA. Changes in exoIII stops were minimally detectable on nucleosomal templates bearing mutations of both the eG and eH HNF3 sites (Figure 3A, lanes 10–12), which is consistent with the diminished NS-A1 DNase I footprint on the eG⁻/eH⁻ cores. We conclude that high-affinity binding by HNF3 near the dyad, coincident with NS-A1 site occupancy on the upstream side of the nucleosome, results in a shift in upstream exoIII protection on N1 sequence particles.

No discrete shifts in downstream N1 core protection were seen upon HNF3α binding, although a novel exoIII stop was generated on nucleosomes at site eX, where new exoIII stops are also seen on free DNA (Figure 3B, lanes 1–5). Given that HNF3 gives a weak DNase I footprint on free DNA at the eX site (data not shown), that the eX

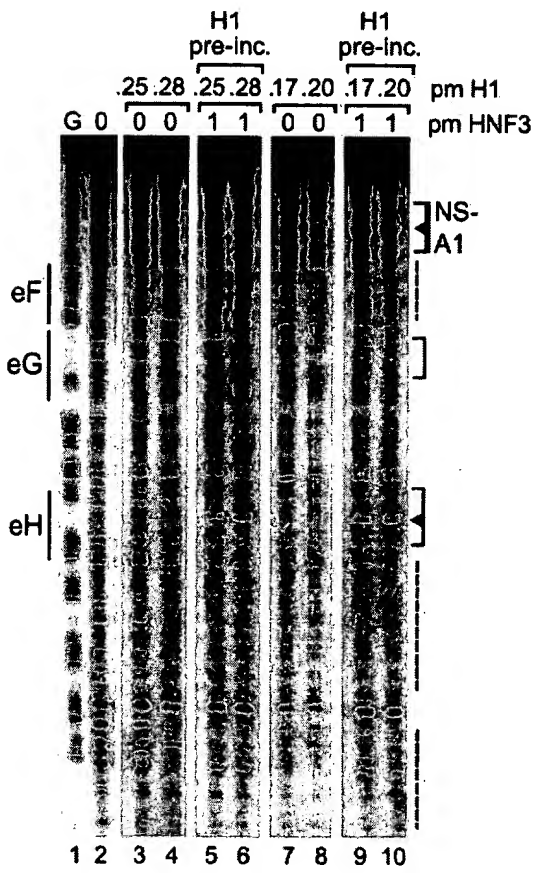

Fig. 4. Displacement of histone H1 by HNF3. DNase I footprinting of wild-type albumin enhancer nucleosome cores with the designated picomole (pm) amounts of liver (lanes 3–6) or thymus (lanes 7–10) histone H1 and HNF3. N1 sequence core particles were incubated with H1 for 40 min (lanes 5 and 6) or pre-incubated with H1 for 20 min followed by addition of HNF3 for an additional 20 min (lanes 7–10). All lanes are from the same gel. Lanes 1 and 2 were exposed to film for 2 days whereas lanes 3–10 were exposed for 3 days to normalize for the general inhibition of DNase cleavage by histone H1 (e.g. see Ura *et al.*, 1996).

site is exposed on the linker segment of core particles in position A (Figure 3C), and that no changes were observed in downstream boundaries for positions B and C (Figure 3B, lanes 4 and 5), we believe that HNF3 selectively binds the exposed eX site on the subset of nucleosomes in position A. The eX site is occupied by an unknown factor *in vivo* whose footprint characteristics do not resemble those of HNF3 (McPherson *et al.*, 1993). In conclusion, asymmetric occupancy of the NS-A1 site on one side of the nucleosome core is associated with asymmetric protection of the particles from exoIII.

Interestingly, intermediate amounts of liver histone H1 led to several changes in the upstream exoIII protections of N1 cores which were identical to those created by HNF3α (Figure 3A, lanes 7–9). As with HNF3α, an intermediate amount of histone H1 did not change downstream nucleosome boundaries (Figure 3B, lane 7). Thus, linker histone causes nucleosomal protection similar to HNF3α in the vicinity of NS-A1, as detected by exoIII digestion of potential linker DNA.

Displacement of linker histone by HNF3

Considering that our *in vitro* DNase I footprinting data showed that both histone H1 and HNF3α can bind the NS-A1 region of the N1 sequence nucleosome cores, and that histone H1 exchanges between sites at the salt concentrations in our assays (Caron and Thomas, 1981), we asked whether HNF3α could displace the linker histone in a sequential binding experiment. Histone H1 was incubated with wild-type albumin enhancer N1 sequence cores for 20 min, and then HNF3 was added for a further 20 min incubation. As seen in Figure 4 (lanes 3 and 4), we used amounts of liver H1 that elicited selective protection at the NS-A1 site and elsewhere (dashed lines and bracket at side of Figure 4). Subsequent addition of HNF3 led to nearly complete occupancy of NS-A1, as evidenced by further site protection and the characteristic hypersensitive cleavage (Figure 4, lanes 5 and 6). Thus, HNF3 displaced pre-bound H1. Because the liver histone H1 preparation was partially degraded and apparently enriched in globular domain fragments (Allan *et al.*, 1980), we repeated the displacement experiment with calf thymus histone H1 from a commercial source. Similar results were obtained as with liver H1 (Figure 4, lanes 7 and 8). Subsequent addition of HNF3 led to nearly complete NS-A1 site protection and strong DNase I hypersensitivity (Figure 4, lanes 9 and 10), as when the liver H1 was pre-bound. The DNase pattern of the 'H1-displaced' nucleosomes resembled that of nucleosomes bound by HNF3 alone and not free DNA. We conclude that HNF3 can displace a saturating amount of histone H1 from the nucleosome core particle.

Lack of nucleosomal DNA compaction by HNF3

To investigate whether HNF3 might affect DNA compaction on the nucleosome like linker histone, we compared the ability of HNF3 and H1 to protect DNA extending from the particles in a micrococcal nuclease (MNase) digestion assay. Linker histone binding should give rise to a digestion intermediate called the chromatosome (Simpson, 1978) due to transient protection of linker DNA which otherwise extends from the core particles. We found that, as expected, nucleosome cores alone gave rise to an ~146 bp terminal MNase digestion product, whereas prior binding of histone H1 led to a 'chromatosome stop' of ~166 bp (Figure 5, lanes 7 and 8). By contrast, concentrations of HNF3α which led to NS-A1 site occupancy either failed to give rise to a chromatosome-sized product or, in some experiments, gave rise to a faint band suggesting a very small amount of chromatosome-sized material (Figure 5, lanes 9–11). We conclude that despite their nucleosome binding similarities, HNF3 protein does not compact potential linker DNA on the nucleosome, as does linker histone.

Adding linker histone properties to HNF3

To establish definitively whether or not HNF3 can bind to a functional linker histone site on the nucleosome core, we made variant HNF3 proteins which contain basic amino acid residues that confer upon linker histone the capacity to compact DNA on the nucleosome. The crystal structure analysis of linker histone H5 revealed that side chains of lysines 40 and 52 and arginines 42 and 94 extend away from the winged-helix domain, on the side

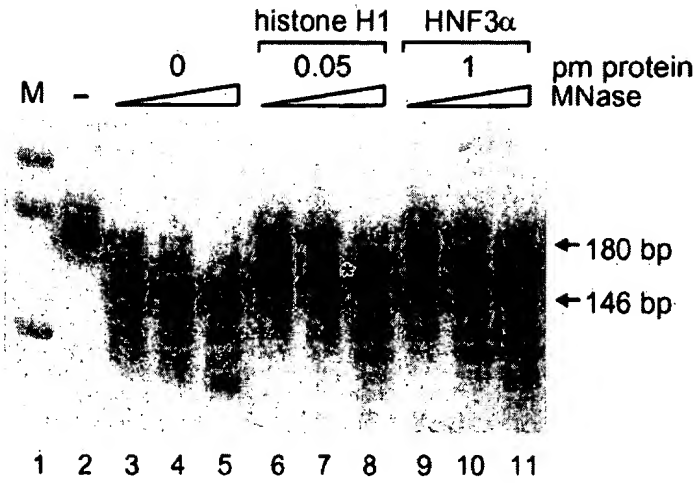

Fig. 5. HNF3 lacks the ability to compact DNA on the nucleosome. Chromatosome assay: after the designated amounts of liver histone H1 or HNF3α were incubated with uniformly labeled albumin enhancer nucleosome cores, increasing amounts of MNase were added to each binding reaction. DNA was purified and analyzed on native 8% polyacrylamide gels. The positions of the initial 180 bp DNA and 146 bp terminal digestion product are shown by arrows; the chromatosome intermediate observed after binding by histone H1 is indicated by an asterisk. Lane M, φX174 *Hae*III DNA length markers.

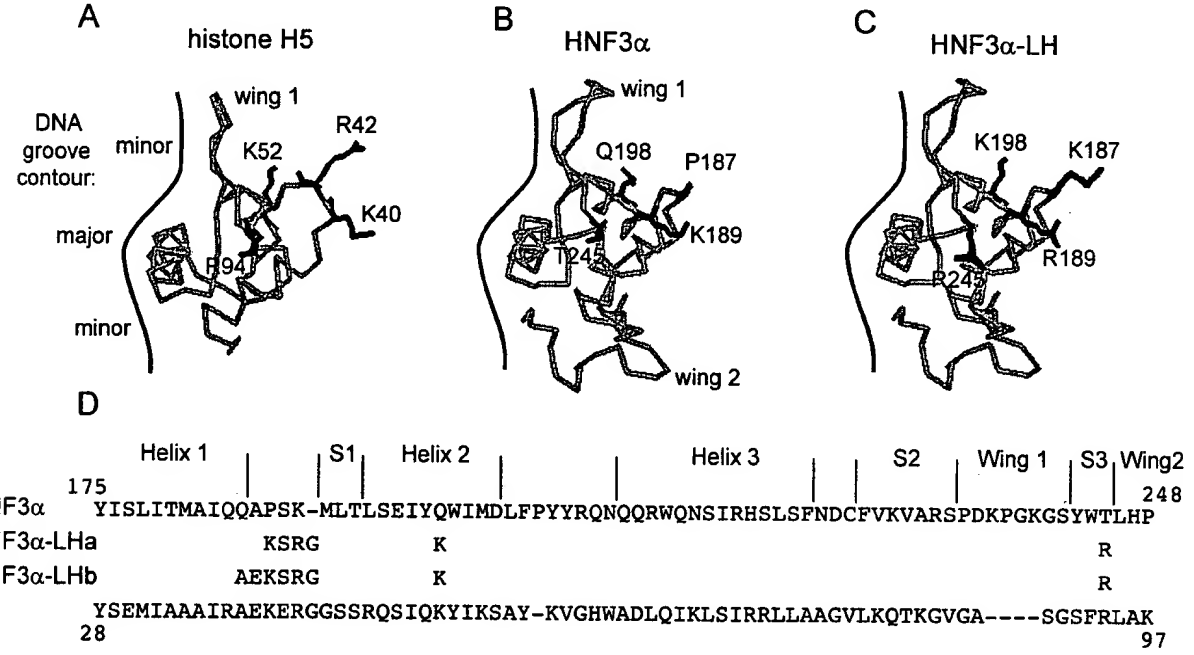

Fig. 6. Adding linker histone characteristics to HNF3α. (**A**) Structures of linker histone (Ramakrishnan *et al.*, 1993), HNF3 (Clark *et al.*, 1993) and HNF-LH variant proteins (structure of the latter by conjecture). Critical amino acids are numbered and shown with their functional groups in black. The positions of wings 1 and 2 are indicated. The curved line to the left of each figure indicates the approximate surface contour of the DNA helix to which the protein can bind, based on the structure of the HNF3α–DNA co-crystal (Clark *et al.*, 1993). (**B**) Amino acid sequences of most of the winged-helix domains of HNF3α and histone H5 are shown. The numbers at the beginning and end of each sequence indicate positions relative to the entire protein. The HNF3-LHa and -LHb variants contain substitutions of the residues shown. Dashes are introduced where amino acid gaps permit the best structural alignment of HNF3 and H5. Structural domains are indicated above the amino acid sequences. S = β-sheet.

of the globular domain which is opposite to that which contacts DNA (Ramakrishnan *et al.*, 1993) (Figure 6A). These residues are conserved in the structure of histone H1 (Cerf *et al.*, 1994). Goytisolo *et al.* (1996) recently showed that converting these four amino acids in linker histone H5 to alanine resulted in a globular domain peptide which still bound nucleosome cores, but failed to compact DNA on the cores in a chromatosome assay. Thus, the four basic amino acids contribute to the nucleosome compaction properties of linker histone, as assessed by chromatosome formation. HNF3α and most other winged-helix transcription factors have a lysine residue at position 189, which is structurally similar to that of the critical Lys40 in H5 (Clark *et al.*, 1993), but no other basic amino acids occur in HNF3 at positions similar to those required for nucleosomal DNA compaction by linker histone (Figure 6B and D). We therefore used molecular modeling to design two variant HNF3 molecules, HNF3-LHa and

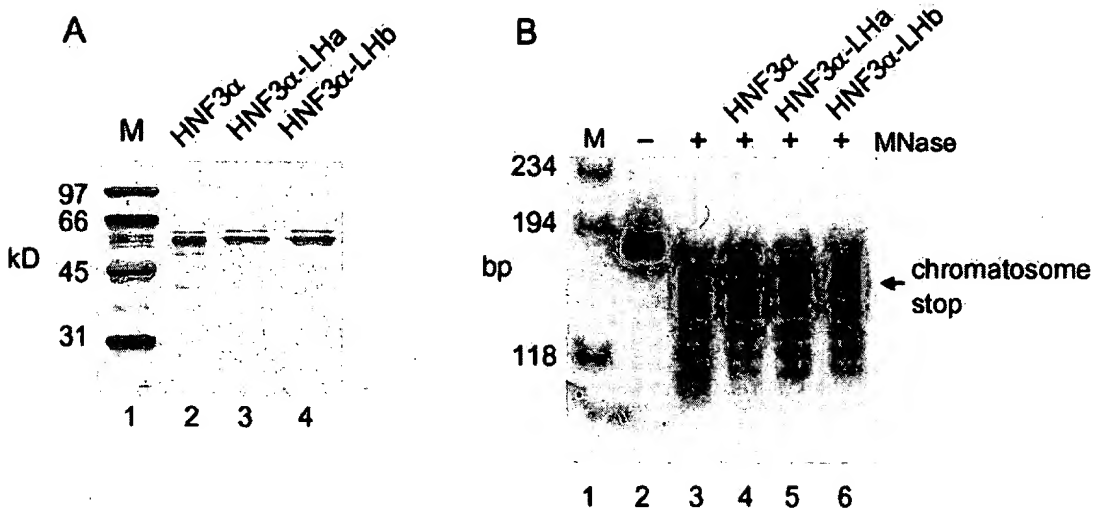

Fig. 7. The HNF3-LHb variant creates chromatosomes. (A) SDS–PAGE analysis of purified HNF3, HNF3-LHa and -LHb. (B) Chromatosome assays as in Figure 5.

HNF3-LHb, each of which has a total of four lysine and arginine residues at similar positions as in the linker histone globular domain (Figure 6C). We also converted amino acids in the vicinity of the new Lys187 and Arg189, in an effort to create a loop between α-helix 1 and β-sheet 1 similar to that in linker histone, with the HNF3-LHb variant containing two more changes than HNF3-LHa (Figure 6D). The HNF3-LH variants were overexpressed in *Escherichia coli*, purified (Figure 7A) and used in chromatosome assays as described above for linker histone. To normalize for extents of MNase digestion between different samples, we compared midpoints in the digestion curves.

Whereas wild-type HNF3 induced a barely detectable level of chromatosome product, the HNF3-LHb variant reproducibly yielded chromatosome-sized material (Figure 7B, compare lanes 4 and 6). In multiple experiments with different nucleosome core preparations, the HNF3-LHa variant gave rise to chromatosome-sized products at a level which was intermediate between that seen with HNF3 and HNF3-LHb (Figure 7B, lane 5). We conclude that HNF3 can bind to a functional linker histone site on the nucleosome core, and that basic residues specific to linker histone's globular domain can confer upon HNF3 the ability to compact DNA on the particle.

Occupancy of the NS-A1 site in vivo

To address whether the NS-A1 site is occupied selectively in active or inactive chromatin, we employed an *in vivo* footprinting protocol on the albumin enhancer in various tissues. We recently showed that the albumin enhancer is occupied by transcription factors at the eF and eG sites in pre-hepatic endoderm which expresses HNF3, and that neither of these sites are occupied in embryo head cells, which do not express HNF3 and in which the albumin gene is inactive (Gualdi *et al.*, 1996). To investigate NS-A1 site occupancy, we treated cells from these tissues with dimethylsulfate (DMS) and used ligation-mediated PCR to map guanosine residues which were protected from DMS modification. As seen in Figure 8A, guanosines were clearly protected at the NS-A1 site in the hepatic precursors, but not in the embryo head cells. We also performed the footprinting assay by perfusing intact liver of adult mice with DMS. Although the adult liver samples were contaminated with ~40% non-parenchymal cells, protection of two Gs at NS-A1 was evident (Figure 8B). We conclude that the NS-A1 site is occupied on the albumin enhancer N1 particle specifically in active or potentially active chromatin.

Discussion

We have shown that the winged-helix structure, which constitutes the primary nucleosome-binding domain of linker histones H1 and H5, also confers nucleosome binding upon a transcription factor. As for all other known transcription factors (Adams and Workman, 1995), high-affinity free DNA target sites for HNF3α, such as the albumin enhancer eG and eH sites, require higher concentrations of protein to be occupied on nucleosome cores. By contrast, the NS-A1 site becomes occupied by the same concentration of HNF3α on both free DNA and nucleosome cores. Although Li and Wrange (1993) and Vettese-Dadey *et al.* (1994) also found that certain transcription factors bound better near the edge of a nucleosome core than near the dyad axis, in those cases the proteins had markedly higher affinities for their binding sites on free DNA than nucleosomes. Binding of HNF3 to the NS-A1 site on nucleosome cores is selective, in that it occurs at the same HNF3 concentration required to bind the eH site, whereas on free DNA, NS-A1 site binding is not distinguished from non-specific HNF3 occupancy on the template. Apparently, HNF3 recognizes a novel conformation of DNA and/or histone on the nucleosome core which may be mimicked by excess HNF3α bound non-specifically to free DNA. We found that NS site binding by the winged-helix domain of HNF3 is weak, and is enhanced by the amino- and carboxy-terminal domains. Interestingly, the latter domains confer transcriptional activation on HNF3 (Pani *et al.*, 1992).

The NS-A1 site described here, TGTCAGC, possesses only five out of 7 bp similarity to the HNF3 target site

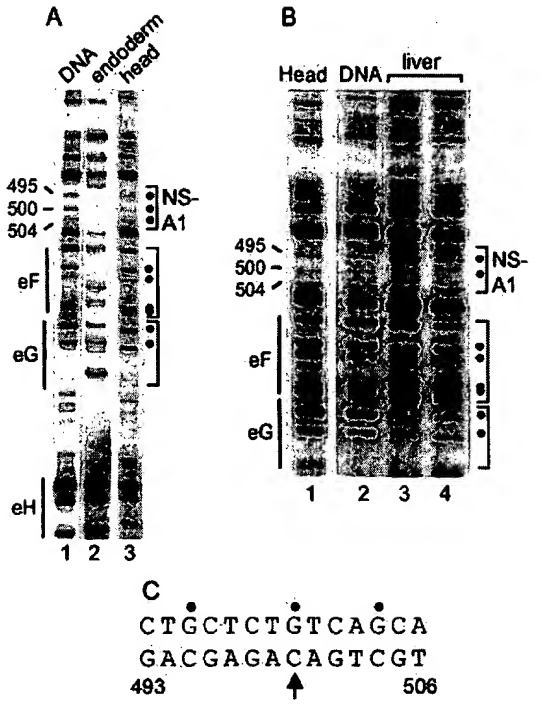

Fig. 8. *In vivo* footprinting of an HNF3 nucleosome structural site. (**A**) Hepatogenic endoderm from 9.5 day mouse embryos and head tissues was dispersed and treated with DMS, and the sites of DMS protection were mapped by LM-PCR. Control reactions ('DNA') consisted of mouse liver genomic DNA treated with DMS *in vitro*. The relevant enhancer nucleotide positions are shown on the left of each panel, and the G residues protected at the bracketed NS-A1, eF and eG sites are shown by dots on the right. (**B**) A mouse liver was perfused *in situ* with a DMS solution and different liver fragments were prepped and analyzed by LM-PCR (lanes 3 and 4). (**C**) Nucleotide sequence of the NS-A1 region footprint and positions of *in vivo* protection. The arrow indicates the position of DNase I hypersensitivity when HNF3 binds N1 sequence nucleosome cores *in vitro*.

consensus, TGTTTGC, when aligned by the position of the DNase I-hypersensitive site within the footprint. The NS-A1 site substitution mutation, CTAGCTA, deviates completely from the HNF3 consensus and prevents HNF3 binding (Figure 1C); thus, the protein recognizes both DNA sequence and nucleosome structure on the core particles. HNF3 induces DNase I hypersensitivity within its footprint because the protein bends DNA towards itself, slightly widening the minor groove on the opposite side (Clark *et al.*, 1993; Pierrou *et al.*, 1994). HNF3 may be able to bind nucleosome sites which deviate from the consensus because the DNA is already bent on the nucleosome substrate. Our observation that DNase I can cleave within the NS footprint on N1 particle cores is consistent with the winged-helix domain fitting into the side of the nucleosome core (Pruss *et al.*, 1995). This would expose the other face of the HNF3-bound DNA segment to solution, allowing DNase I cleavage.

The binding of HNF3 to one side of the nucleosome core is similar to the asymmetric binding of linker histones H1 and H5 on nucleosome cores of the 5S rRNA gene (Hayes and Wolffe, 1993; Hayes *et al.*, 1994; Hayes, 1996). While linker histone could not be footprinted with DNase I on the 5S rRNA cores, linker histone could be detected at a specific location of DNA by cross-linking and asymmetric protection from MNase. We found that linker histone bound the NS-A1 region, in addition to other regions of the albumin enhancer cores, indicating similarities in the way in which linker histone and HNF3 recognize local nucleosome structure. However, the ability of H1, but not HNF3, to elicit DNase I protection of the NS-A1 region on the NS-A1 site mutant nucleosomes indicates that H1 binding there is more dependent on local nucleosome structure than on sequence. While heterogeneity in the albumin enhancer core positions precluded a precise mapping of the structural position of the NS-A1 site, based on the changes in exoIII protection upon HNF3 binding, the NS-A1 site appears to be within about a helical turn of the edge of nucleosome core position B (Figure 3C).

Wolffe and Pruss (1996) postulated that HNF3α binding to the eG or eH site might displace histone H1. Although we failed to observe specific H1 protection of the eG and eH sites, HNF3 displaced H1 that was pre-bound to the NS-A1 site on nucleosome cores. By contrast, H1 inhibits the transcription factors USF and GAL4 from binding to nucleosomes (Juan *et al.*, 1994), demonstrating a distinct behavior of HNF3. The primary functional difference between linker histone and HNF3 binding to nucleosomes, in terms of only linker histone being able to compact DNA on the particle, is due in part to the four basic amino acids within the winged-helix motif being present solely on the linker histones (Goytisolo *et al.*, 1996). We demonstrated that placing these basic residues at structurally similar sites on HNF3, in combination with changes in certain adjacent amino acids, is sufficient to confer upon HNF3 the ability to protect additional DNA on the nucleosome core particle from MNase digestion, a property previously observed only upon linker histone binding. Although the exact mechanism by which linker histone compacts DNA on the nucleosome has been debated (Hayes *et al.*, 1996), the chromatosome assay clearly demonstrates that HNF3 can occupy a functional linker histone site on the nucleosome. Further studies will be required to understand how HNF3 binding at sites like NS-A1 affects higher-order chromatin compaction.

In development, transcription factors containing the winged-helix domain play critical roles in all metazoans in which they have been discovered. *In vivo* footprinting analysis showed that the NS-A1 site on the albumin enhancer is occupied in cells where the enhancer is active or potentially active, but not in cells where the enhancer remains silent in development. The results provide examples of where NS-A1 site occupancy, apparently by HNF3, is associated specifically with gene activation. We speculate that the binding of HNF3 to nucleosomal sites, coupled with the factors' inability to compact nucleosomal DNA, might help target genes assume an open configuration, primed for other transcription factors to bind and activate gene expression. While it is not yet known how many of the 80 winged-helix transcription factors which have been identified (Kaufmann and Knöchel, 1996) possess a nucleosome core-binding activity like HNF3, the very high amino acid sequence similarity between HNF3 and other fork head family members suggests that the properties described here are likely to be common among these critical developmental regulatory proteins.

Materials and methods

Protein purification

HNF3α full-length protein was expressed from DNA of the coding sequence (Kaestner *et al.*, 1994) assembled from PCR-generated fragments of a mouse genomic clone of the HNF3α gene (A.Grant and K.S.Zaret, unpublished). The mouse HNF3α coding sequence was inserted into the pET28b plasmid vector (Novagen) and overexpressed in and purified from *E.coli* with a six-histidine amino-terminal tag as described (Zaret and Stevens, 1995). The HNF3-LHa and -LHb site-directed mutants were made by overlap extension PCR (Ho *et al.*, 1989) with the modification of Mikaelian and Sergeant (1992). We first created the T245R mutation and then used the altered plasmids for a second round of mutagenesis to introduce other changes. All sequence changes and regions cloned by PCR were confirmed by DNA sequence analysis. The HNF3α DNA-binding domain was purified from *E.coli* as described for the HNF3γ protein (Clark *et al.*, 1993). The molecular mass of full-length HNF3α was ~54 500 Da; that of the DBD was ~13 700 Da. Histone H1 was purified from a pig liver as described by Croston *et al.* (1991). SDS–PAGE analysis of the final product indicated that ~30% of the protein was the full-length size of 21–23 kDa and the rest was partially degraded fragments of discrete sizes. The molarity of H1 in binding reactions was based on the mass of protein and the assumption that the protein was full length. Because subfragments of H1 containing the winged-helix globular domain retain nucleosome-binding capacity (Allan *et al.*, 1980), the molarities shown in the figures are several-fold underestimates of the concentrations of molecules competent to bind nucleosomes. We also used calf thymus H1 from Boehringer Mannheim.

Nucleosome core preparation

Nucleosome cores were prepared as described by McPherson *et al.* (1996). Briefly, end-labeled or internally labeled PCR fragments of specific DNA, along with a 10-fold mass excess of carrier DNA, were assembled onto purified core histone proteins by salt–urea gradient dialysis, and the assembled cores were purified from free DNA by glycerol gradient sedimentation. Nucleosome cores were dialyzed against a solution of 10 mM Tris–HCl pH 8.0, 1 mM 2-mercaptoethanol, concentrated, and stored at 4°C. The albumin enhancer DNA fragment corresponds to positions 472–651 of the –10 kb enhancer and was created by PCR with end-labeled primers, or PCR was performed in the presence of [α-^{32}P]dATP to generate uniformly labeled DNA for chromatosome assays. Plasmid templates contained either the wild-type enhancer (Liu *et al.*, 1991), the enhancer with the NS-A1 sequence from positions 495 to 505 changed to TAAGCTAGCTA or the enhancer with clustered point mutations of both the eG (M2; Liu *et al.*, 1991) and eH (M4; Jackson *et al.*, 1993) HNF3-binding sites.

Binding reactions and enzymatic assays

Binding reactions for the electromobility shift, DNase I, exoIII and MNase assays were carried out in 20 µl volumes containing a nucleosome concentration of 5 ng/ml or ~20 nM, corresponding to 0.42 pmol per reaction (0.04 pmol of specific template, given carrier particles). Binding reactions with free DNA contained identical molar amounts of templates. We previously showed that the nucleosomes were stable under these conditions (McPherson *et al.*, 1996) and the data in this report show that HNF3 binding did not induce nucleosome instability, which would be evident as having generated the same mobility as free DNA–HNF3 complexes in Figure 2B, causing exonuclease penetration of the particles in Figure 3, or enhancing MNase digestion of the particles in Figure 5. Purified HNF3 was thawed on ice and diluted in 20 mM HEPES pH 6.5, 5 mM dithiothreiotol (DTT), 1 mM $MgCl_2$, 100 mM KCl, 20% glycerol, 0.1% NP-40 and 0.25 mg/ml bovine serum albumin (BSA). HNF3 was added to the nucleosomes in final buffer conditions of 10 mM Tris–HCl pH 7.5, 1 mM $MgCl_2$, 5 mM DTT, 40 mM KCl, 0.5% glycerol, 3 mg/ml BSA and 1% Ficoll, and the reactions were incubated at 21–24°C for 30 min. For the electromobility shift assays, binding reactions were loaded onto 4% polyacrylamide gels in 0.5× Tris–borate–EDTA buffer. The quantitations described in the text are the average of two separate experiments. For DNase I assays, 1 µl of DNase I (diluted in 20 mM $MgCl_2$, 1 mM $CaCl_2$) was added to the binding reactions to a final concentration of 0.025–0.075 µg/ml and 0.5–2 µg/ml for free and nucleosomal DNA, respectively, for 1 min at 21–25°C. For exoIII analysis, 0.5 and 5 U of exoIII (diluted in 66 mM Tris–HCl pH 8.0, 0.66 mM $MgCl_2$, 50% glycerol) was added to binding reactions containing free and nucleosomal DNA respectively, followed by incubation for 1 min at 21–25°C. All reactions were stopped by addition of an equal volume of stop buffer (30 mM EDTA, 0.1% SDS, 50 µg/ml tRNA, 0.35 M NaCl), extracted with phenol/$CHCl_3$ (1:1), then $CHCl_3$ alone, and ethanol precipitated. The DNA was resuspended in formamide loading buffer and separated on 6% polyacrylamide–7 M urea sequencing gels. For MNase analysis, 0.025–0.15 U of MNase (diluted in 5 mM Tris–HCl pH 7.5, 25 µM $CaCl_2$) was added to the binding reactions, followed by incubation for 5 min at 21–25°C. Reactions were stopped by addition of EGTA to 2.5 mM, extracted with phenol/$CHCl_3$ and $CHCl_3$, and ethanol precipitated. The DNA was resuspended in 10 mM Tris–HCl pH 8.0, 1 mM EDTA and separated on 8% non-denaturing polyacrylamide gels.

In vivo and in vitro DMS footprinting

Embryo cell isolation, liver perfusion, DMS treatment, DNA purification and LM-PCR analysis were performed as described by McPherson *et al.* (1993) and Gualdi *et al.* (1996). The control tissue for LM-PCR was from heads of 12.5 day mouse embryos.

Acknowledgements

We thank Sergei Grigoryev, Rachel Horowitz, Art Landy, Venki Ramakrishnan, John Sedivy, Chris Woodcock and Alan Wolffe for valuable discussions, and Carmen González for help in preparing the manuscript. The work was supported by postdoctoral fellowships from the NIH (L.A.C. and C.E.M.) and the Ligue Contre Le Cancer (P.B.), and by grants GM47903 and GM36977 from the NIH (K.S.Z.).

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Received August 11, 1997; revised and accepted October 23, 1997